

Hsp70 positively regulates porcine circovirus type 2 replication in vitro



Jie Liu^a, Juan Bai^a, Lili Zhang^a, Zhihua Jiang^b, Xianwei Wang^a, Yufeng Li^a, Ping Jiang^{a,*}

^a Key Laboratory of Animal Diseases Diagnostic and Immunology, Ministry of Agriculture, College of Veterinary Medicine, Nanjing Agricultural University, Nanjing, Jiangsu 210095, China

^b Department of Animal Sciences, Washington State University, Pullman, WA 99164-6351, USA

ARTICLE INFO

Article history:

Received 3 July 2013

Returned to author for revisions

26 July 2013

Accepted 22 August 2013

Available online 18 September 2013

Keywords:

Porcine circovirus type 2

Heat shock response

Hsp70

NF- κ B

Caspase-3

ABSTRACT

The Hsp70 chaperone plays a central role in multiple processes within cells. Porcine circovirus type 2 (PCV2) is the essential causal agent of post-weaning multisystemic wasting syndrome (PMWS), which has spread worldwide. But the mechanism of PCV2 replication remains poorly understood. In this study, we firstly found the positive effect of heat stress on the replication of PCV2 in the continuous porcine monocytic cell line 3D4/31. Downregulation of Hsp70 by the specific chaperone inhibitor Quercetin or RNA interference and upregulation of Hsp70 by expression from a recombinant adenovirus showed that Hsp70 enhanced PCV2 genome replication and virion production. A specific interaction between Hsp70 and PCV2 Cap was confirmed by colocalization by confocal microscopy and co-immunoprecipitation. Furthermore, the NF- κ B pathway was activated and caspase-3 activity was reduced when Hsp70 was overexpressed in PCV2-infected 3D4/31 cells. These data suggested that Hsp70 positively regulated PCV2 replication, which being helpful for understanding the molecular mechanism of PCV2 infection.

© 2013 Elsevier Inc. All rights reserved.

Introduction

Porcine circoviruses (PCVs) are members of the family *Circoviridae*, genus *Circovirus*, which are the smallest nonenveloped, single-stranded, circular DNA viruses that replicate autonomously in mammalian cells. Two types of PCVs have been characterized to date, PCV1 and PCV2 (Kim et al., 2006). PCV2 has a genome size of 1.7 kb. Four PCV2 viral proteins have been identified: a replication protein that is associated with viral replication (Mankertz et al., 1998), an ORF3-encoded protein involved in cell apoptosis (Liu et al., 2005), a capsid (Cap) protein responsible for immune response (Nawagitgul et al., 2000), and ORF4 protein that is involved in suppressing caspase activity and decreasing CD4 (+) and CD8 (+) T lymphocytes during PCV2 infection (He et al., 2012). PCV2 is the pathogen that causes PCV2-associated diseases including post-weaning multisystemic wasting syndrome (PMWS) (Allan and Ellis, 2000; Allan et al., 2004; Segales et al., 2005, 1997), reproductive failure, porcine respiratory disease complex, granulomatous enteritis, necrotizing lymphadenitis (Chae, 2005), and acute pulmonary edema (Cino-Ozuna et al., 2011). However, not all pigs infected with PCV2 will develop PMWS. Even if PMWS occurs, the severity of the disease differs at different pig farms. Infection of pigs with PCV2 and other infectious/noninfectious triggers are required for PMWS to develop (Grau-Roma et al., 2011; Patterson and Opriessnig, 2010). Several

studies have linked PMWS to management measures, presence of concurrent viral infections, immune system stimulation, nutrition, male castration and lower piglet weight at weaning (Grau-Roma et al., 2011). However, the mechanism of replication and pathogenesis of PCV2 remains poorly understood.

Host cell responses to metabolic or environmental stresses are generally mediated by inducing synthesis of a selected family of proteins, collectively known as stress proteins or heat-shock proteins (Hsps) (Morimoto, 1991). They are involved in many viral life cycle activities, such as transcription, cellular transformation, viral genome replication, and increased virion assembly (Sullivan and Pipas, 2001). Among them, the ubiquitous chaperone family of 70 kDa HSPs (Hsp70) plays a central role in protein homeostasis and protection against proteotoxic stresses. It not only surveys the folding status of proteins under stress conditions, but is involved in the regulation of fundamental cellular processes such as signal transduction, cell cycle regulation, apoptosis, and innate immunity (Mayer, 2010; Mayer and Bukau, 2005). Evidence is growing that Hsp70 plays important roles in the replication of many viruses, such as herpesvirus (Diaz-Latoud et al., 1997; Santoro, 1994), vaccinia virus (Jindal and Young, 1992), West Nile virus and measles virus (Nagy et al., 2011). An increase in Hsp70 expression confers protection to cells against vesicular stomatitis virus (Demarco and Santoro, 1993) and influenza virus (Hirayama et al., 2004) infection. But there is no information concerning the production of the heat shock response (HSR) induced by heat stress and the effects of Hsp70 on PCV2 replication, which may explain the different incidences of PMWS in different pig farms with PCV2 infection. In this study, we firstly found that heat shock could enhance the replication of PCV2 in 3D4/31 cell line. Hsp70 could interact with

* Correspondence to: College of Veterinary Medicine, Nanjing Agricultural University, Nanjing 210095, P.R. China. Fax: +862 584 396 640.
E-mail address: jiangp@njau.edu.cn (P. Jiang).

the Cap protein of PCV2 during the viral replication and the levels of Hsp70 could affect the virus growth in protein and DNA levels. Meanwhile, the NF- κ B pathway was activated and caspase-3 activity was reduced during the virus replication. It indicated that Hsp70 plays important role in the viral transcription, translation and production during the period of PCV2 replication.

Results

Heat shock increased PCV2 infection in 3D4/31 cells

To elucidate the effect of heat stress on 3D4/31 cells infected with PCV2, PCV2-infected cells were grown under physiological conditions or subjected to heat shock for 10 h at 45 °C, then cultured at 37 °C for

72 h. The amount of infected cells was determined by IFA and flow cytometry. Fluorescence measurements showed that the number of infected cells in the heat shock group was much higher than in the control group. The results from three independent experiments clearly showed that heat shock treatment significantly increased the amount of PCV2-infected cells ($P < 0.01$) (Fig. 1A). As shown in Fig. 1B, under physiological conditions, approximately 7.36% of cells were infected (Fig. 1B, middle). However, when cells were treated with heat shock, approximately 39.35% of cells were infected (Fig. 1B, left). These results showed that heat shock treatment increased viral replication efficiency almost five-fold compared with control cells. To corroborate this finding, the level of PCV2 DNA in infected cells was determined by RT-PCR. Compared to the control group, the heat shock group showed a significant increase in PCV2 log₁₀ DNA copies ($P < 0.01$) (Fig. 1C). The virus titer in heat shock group was higher

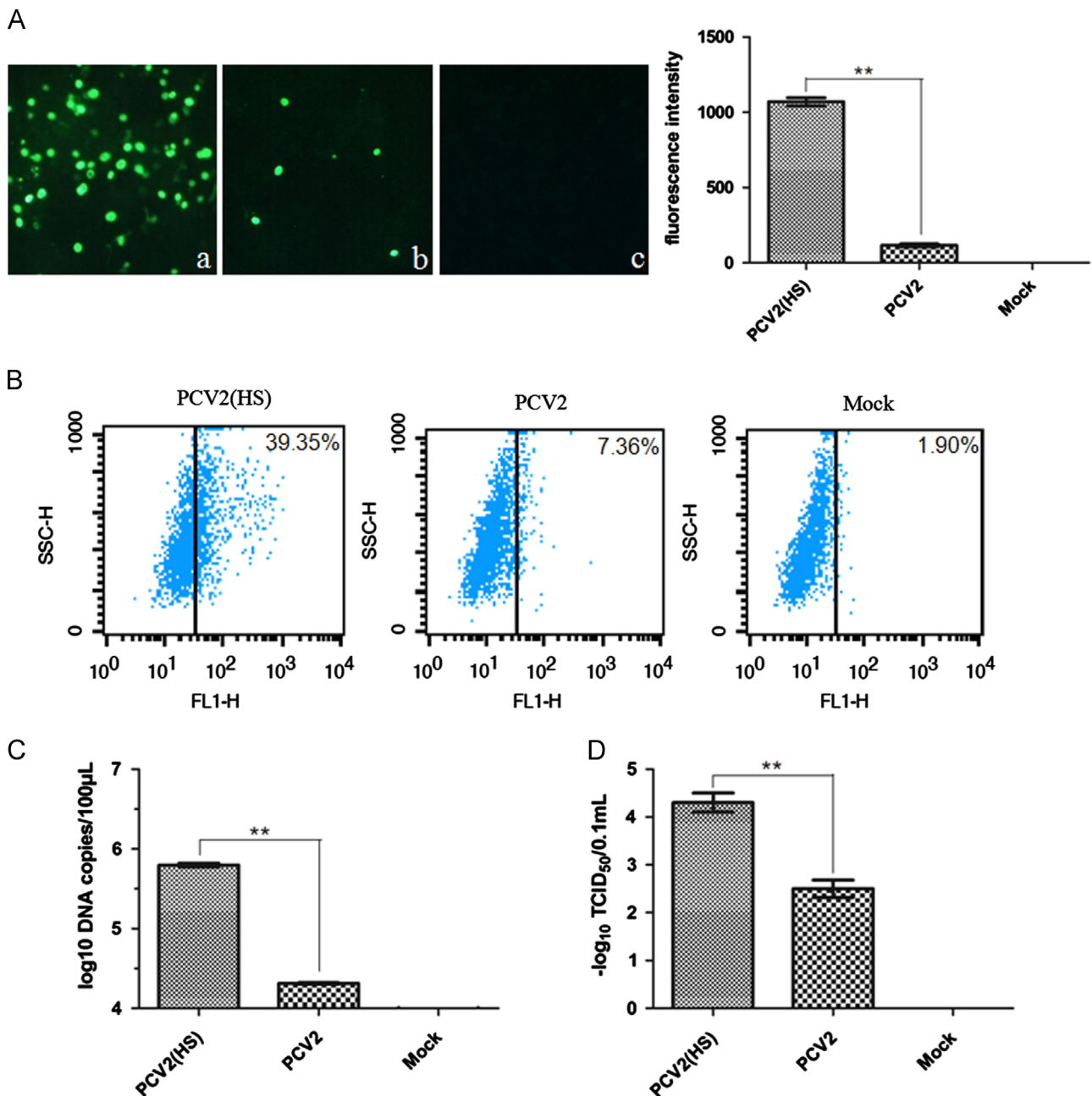


Fig. 1. Heat shock increases PCV2 infectivity. PCV2-infected 3D4/31 cells were heat shocked at 45 °C for 10 h and incubated at 37 °C for 72 h. (A) PCV2-infected 3D4/31 cells were detected by IFA. Fluorescence was measured at 495 nm for excitation and 525 nm for emission. (a) Heat shock treated cells, (b) control cells, (c) non-infected control. (B) Infected cell amounts were determined using flow cytometry for Cap protein-positive cells. Percentile of infection is indicated in panel corners. (C) PCV2-infected cells after heat shock were assayed for viral DNA copy number by real-time PCR. (D) The cell cultures were harvested and PCV2 titers were detected as TCID₅₀.

than that in control group ($P < 0.01$) (Fig. 1D). These data indicated that heat shock contributed to the increase in replication of PCV2 in 3D4/31 cells.

Hsp90, Hsp70 and Hsp27 expression under HS treatment

The HSR involves a marked increase in HSP synthesis, so the expression of Hsp90, Hsp70 and Hsp27 were evaluated. The results showed that Hsp90, Hsp70 and Hsp27 were detected in 3D4/31 cells by Western blotting. However, these HSPs expression were not increased after infected with PCV2. Heating at 45 °C for 5 or 10 h induced increasing amounts of Hsp90, Hsp70 and Hsp27 expression and enhanced PCV2 replication (Fig. 2). These data showed that HS induced expression and accumulation of Hsp90, Hsp70 and Hsp27 in PCV2-infected cells.

Modulation of cellular Hsp70 and effect on PCV2 replication

Whether Hsp70 induction resulting from cellular stress contributes to viral infection is not known. To investigate whether Hsp70 functions during the viral life cycle, the HSR in PCV2-infected cells was blocked by quercetin, an inhibitor of Hsp synthesis, before heat shock. The application of 100 μ M quercetin resulted in a drastic inhibition of Hsp70 expression without causing visible cell damage by comparison to the control, DMSO-treated cells. The results of experiments performed at 72 h after heat shock in Fig. 3A show that quercetin treatment decreased the progression of PCV2 replication as measured by an IFA assay. Western blotting showed the effects of quercetin on synthesis of Hsp70 and PCV2 Cap protein. As shown in Fig. 3B and C, treatment with quercetin dramatically affected the synthesis of Hsp70, viral protein and viral DNA during PCV2 infection. The virus titers detection showed the similar results (Fig. 3D). There was no gross morphological change observed in quercetin treated cells as observed in bright field microscopy (Fig. 3E). These data showed that inhibiting inducible HSPs by quercetin seriously impaired Hsp70 synthesis and production of PCV2.

As quercetin is not a specific inhibitor of Hsp70 but inhibits the synthesis of other heat shock proteins, we performed silencing experiments to investigate more specifically the function of Hsp70 during PCV2 infection. We designed three siRNAs, si1, si2 and si3, and a negative control (namely ns si) targeting the Hsp70 gene. Si1 and si2 had the specific inhibited effect (Fig. 4A). Infected cells were transfected with 400 pmol Hsp70 siRNA for 48 h, and then cells were subjected to normal (control) or heat shock conditions. Hsp70 was efficiently downregulated, as shown in HS-treated cells (Fig. 4B, lanes 1 and 2) compared to control and scrambled siRNA (Fig. 4B, lanes 4 and 5). Hsp90 and Hsp27 were not inhibited (Fig. 4B). These observations were confirmed by the quantification of the cellular Hsp70 protein level (Fig. 4C). Under these

conditions, the knockdown of Hsp70 expression was associated with a significant reduction of the amounts of viral Cap protein (Fig. 4B and C). As expected, the result was similar in virus titers detection showed in Fig. 4D. These results confirmed that the specific inhibition of Hsp70 synthesis led to a reduction of viral protein levels. Hsp70 was involved in the viral transcription, translation and production during PCV2 replication after heat shock.

rAd-mediated gene transfer increased Hsp70 expression and enhanced PCV2 replicative activity

To address our hypothesis that Hsp70 expression enhanced PCV2 replication, we used recombinant adenovirus to transfer Hsp70 into 3D4/31 cells. Briefly, PCV2-infected 3D4/31 cells were inoculated with rAd-hsp70 (500 MOI), and rAd-wt (500 MOI) as a control. The number of PCV2-infected cells was determined by IFA. As shown in Fig. 5A, rAd-hsp70 markedly increased the frequency of fluorescence-stained cells containing PCV2 compared with the control group. The fluorescence value of the rAd-hsp70-treated group was significantly higher than the rAd-wt group ($P < 0.01$) and the PCV2-control group ($P < 0.01$). We also analyzed the effect of rAd-hsp70 on replication of viral DNA in infected cells. At 48 h after inoculation with rAd-hsp70, infected cells were collected and assayed for viral DNA by real-time PCR. Compared to both rAd-wt-treated and PCV2 control groups, the viral DNA content of the rAd-hsp70 treated group showed a notable increase (Fig. 5C) ($P < 0.01$). To further confirm the correlation between Hsp70 overexpression and the induction of PCV2 replication, Western blotting was used to quantitate rAd-mediated Hsp70 and viral protein synthesis. The results showed that rAd-hsp70 successfully transduced an ectogenous gene and expressed the target protein. When Hsp70 was overexpressed by rAd-hsp70 or heat stress, an increase in Cap protein synthesis was observed compared to control groups (Fig. 5B). These data together with virus titers detection (Fig. 5D) indicated that an increase of Hsp70 significantly improved viral protein synthesis and viral production, suggesting that Hsp70 had a positive effect on PCV2 infection.

Hsp70 colocalized with PCV2 Cap protein

To understand the relationship between Hsp70 and Cap protein in the PCV2 life cycle, we performed confocal microscopy for detecting Hsp70 and Cap in PCV2-infected 3D4/31 cells. As shown in Fig. 6, Hsp70 (red) was, on average, distributed evenly in cytoplasm in non-infected cells and PCV2-infected cells under normal conditions. However, when infected cells were subjected to heat shock treatment, hsp70 markedly accumulated in the nuclear zone, the location of the Cap protein (green).

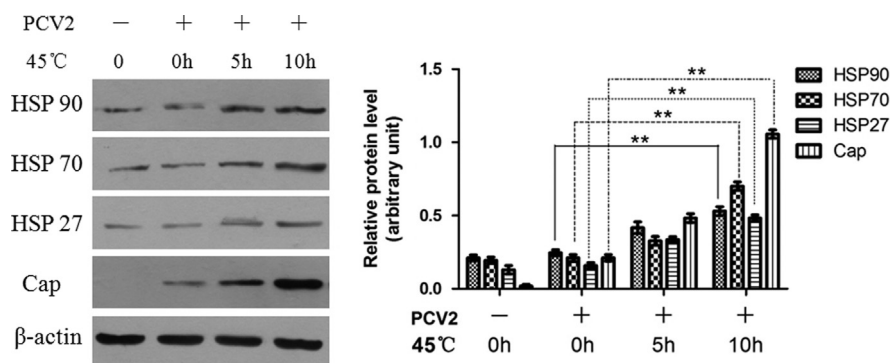


Fig. 2. Changes in the amount of Hsp90, Hsp70, Hsp27 and viral Cap protein in PCV2-infected 3D4/31 in heat shock condition and mock-infected cells by Western blot. The level of relative proteins were quantified by immunoblot scanning and normalized with respect to the amount of β -actin (lower panel).

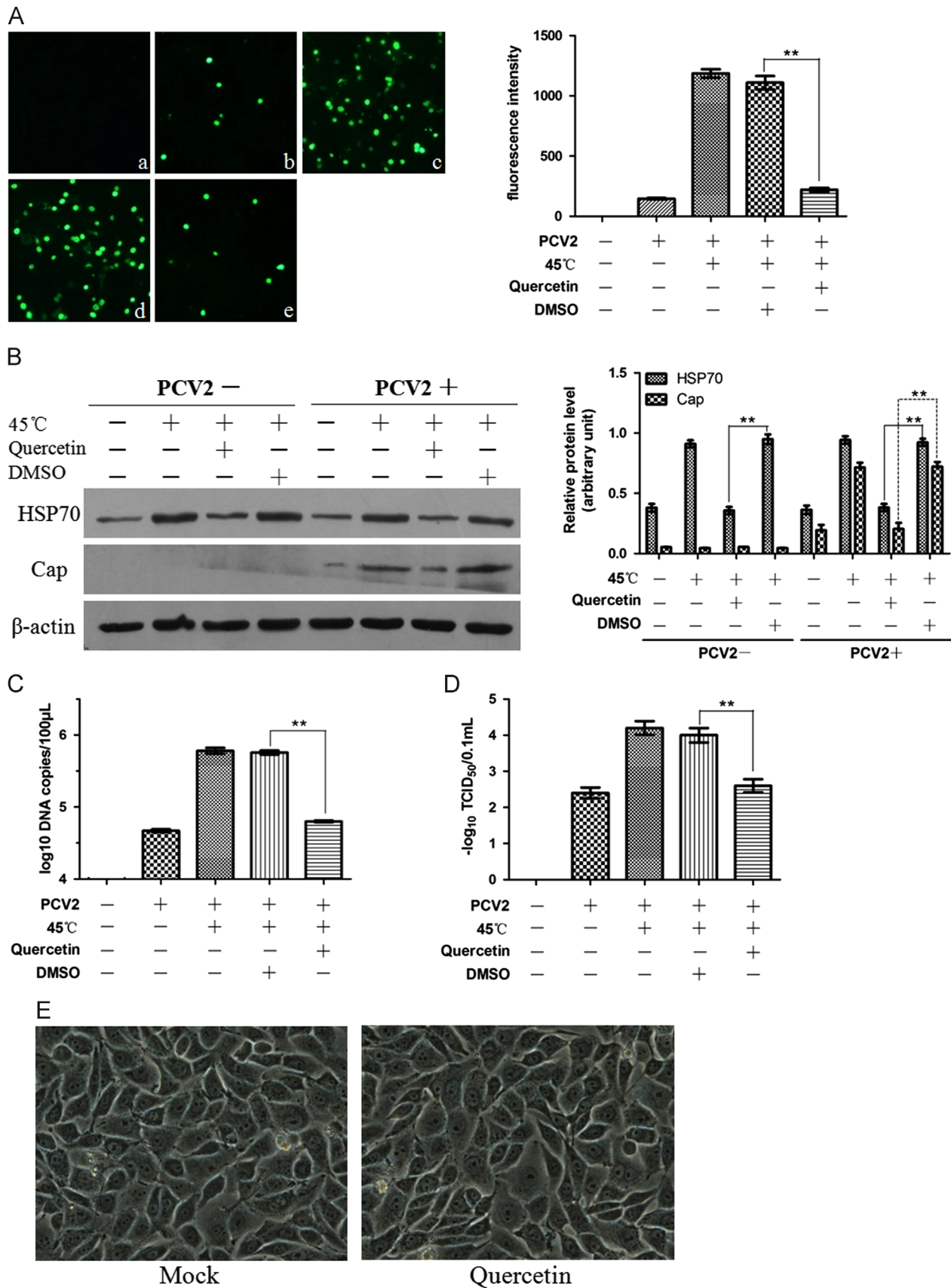


Fig. 3. Effect of Hsp70 inhibition on viral protein synthesis. Before heat shock treatment, PCV2-infected 3D4/31 cells were incubated for 24 h with or without 100 μM quercetin (control with DMSO). (A) PCV2-infected 3D4/31 cells were detected by IFA. (a) Non-infected control, (b) control cells, (c) heat shock treated, (d) DMSO, (e) quercetin. Fluorescence value was measured at 495 nm for excitation and 525 nm for emission. (B) Cells were lysed and cell extracts were analyzed by Western blot using anti-Hsp70, anti-Cap, or anti-β actin antibodies. The level of relative proteins were quantified by immunoblot scanning and normalized with respect to the amount of β-actin (lower panel). (C) PCV2-infected cells after different treatment were assayed for viral DNA copy number by real-time PCR. (D) The cell cultures were harvested and PCV2 titers were detected as TCID₅₀. (E) There was no gross morphological change observed in quercetin treated cells as observed in bright field microscopy.

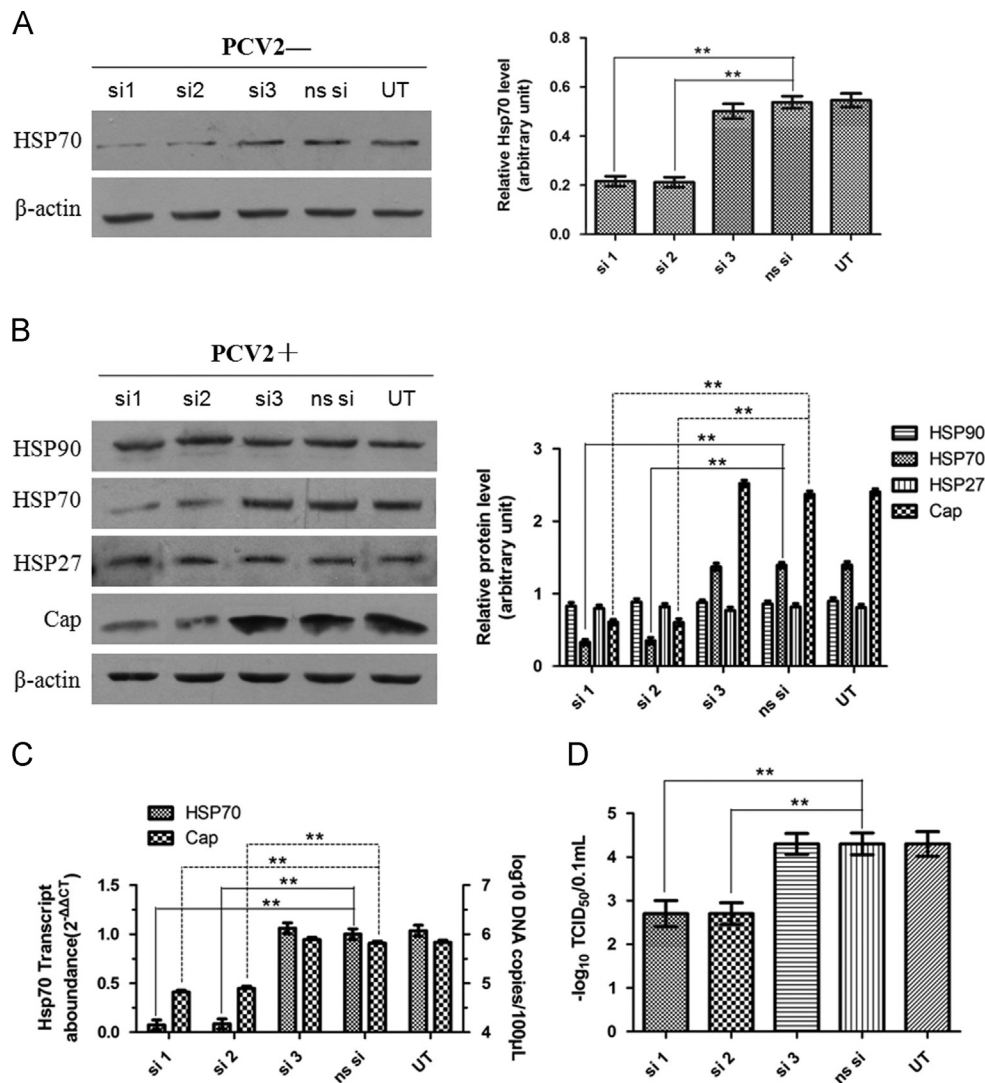


Fig. 4. Knockdown of Hsp70 reduced PCV2 replication. (A) 3D4/31 cells were transfected with siRNA si1, si2, si3 and nonspecific control (ns si) targeting the Hsp70 gene, and incubated for 48 h. The cells were harvested for Western blotting analysis with anti-Hsp70 anti- β -actin antibodies. The levels of Hsp70 was quantified by immunoblot scanning and normalized with respect to the amount of β -actin (lower panel). (B) PCV2 infected 3D4/31 cells were transfected with siRNA si1, si2, si3 and nonspecific control (ns si) targeting the Hsp70 gene, and incubated for 48 h. Then the cells were submitted to heat shock treatment. Cell extracts were analyzed by Western blotting using anti-Hsp70, anti-Hsp90, anti-Hsp27, anti-Cap and anti- β -actin antibodies. The level of relative proteins were quantified by immunoblot scanning and normalized with respect to the amount of β -actin (lower panel). (C) The Hsp70 mRNA and PCV2 Cap gene were detected by quantitative RT-PCR. The level of Hsp70 mRNA was normalized to the level of β -actin mRNA in the same sample. (D) The cell cultures were harvested and PCV2 titers were detected as TCID₅₀.

Hsp70 interacts with Cap protein

To further investigate interaction between Cap and Hsp70, we used heat shock treated PCV2-infected cells or mock-infected cells. Cell extracts were immunoprecipitated with mouse monoclonal antibodies to Hsp70 or Cap. Proteins in the immune complexes were analyzed by Western blotting with anti-Cap or anti-Hsp70. Hsp70 was detected in immunoprecipitates obtained with anti-Cap (Fig. 7, lanes 5 and 6). Reverse experiments showed that Cap protein was immunoprecipitated with antibody against Hsp70 (Fig. 7, lanes 3 and 4), indicating the specificity of the interaction. Thus, these data indicated that Hsp70 interacted with the viral Cap protein in PCV2-infected cells, supporting the hypothesis that this interaction resulted in the co-localization of Hsp70 and Cap.

Elevation of NF- κ B levels during Hsp70-increased PCV2 replication

PCV2 infection activates NF- κ B via I κ B α phosphorylation in PK15 cells (Wei et al., 2008). Since NF- κ B activity is susceptible to regulation by alterations in the intracellular HSP state, the effect of

rAd-hsp70 treatment on PCV2 replication might be associated with an alteration in NF- κ B activity. To determine whether NF- κ B signaling was activated by Hsp70-induced PCV2 replication, the NF- κ B p65 subunit and I κ B α phosphorylation were detected by Western blot at 72 h after rAd-hsp70 inoculation or heat shock treatment. As shown in Fig. 8, PCV2 infection activated NF- κ B p65 protein expression compared with mock-infected cells (lanes 2). When recombinant adenovirus-mediated Hsp70 overexpression was induced in PCV2-infected 3D4/31 cells, using rAd-wt as a negative control, we found that inoculation with rAd-hsp70 led to the accumulation of Hsp70 protein in cells. This markedly induced PCV2 replication. At the same time, the level of p65 protein rose and I κ B α was phosphorylated. These results demonstrated that the NF- κ B pathway was activated when PCV2 replication was induced by Hsp70.

Hsp70 prevents PCV2-induced caspase-3 activation

PCV2 has been shown to induce apoptosis in cultured cells through activation of caspase-8 followed by activation of the

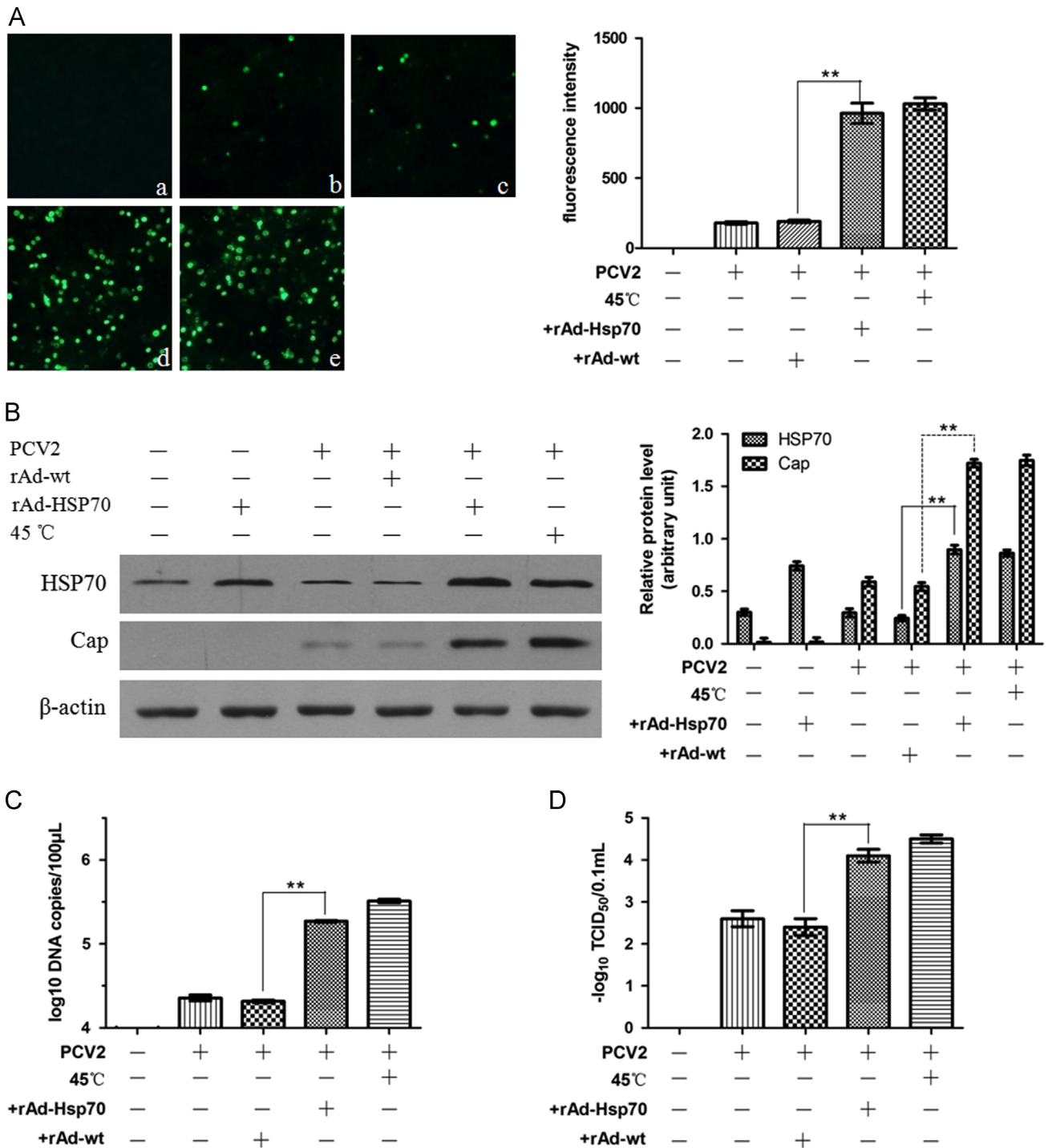


Fig. 5. Effect of recombinant adenovirus-mediated Hsp70 expression on PCV2 replication. 3D4/31 cells were inoculated with rAd-hsp70 after PCV2 infection. (A) PCV2-infected 3D4/31 cells detected by IFA. (a) Non-infected control, (b) control cells, (c) rAd-wt treated, (d) rAd-hsp70 treated, (e) heat shock treated. Fluorescence was measured at 495 nm for excitation and 525 nm for emission. (B) Cells were lysed and cell extracts were analyzed by Western blot using anti-Hsp70, anti-Cap, and anti- β -actin antibodies. The level of relative proteins were quantified by immunoblot scanning and normalized with respect to the amount of β -actin (lower panel). (C) PCV2-infected 3D4/31 cells after rAd-hsp70 inoculation were assayed for viral DNA copy number by real-time PCR. (D) The cell cultures were harvested and PCV2 titers were detected as TCID₅₀.

caspase-3 pathway (Liu et al., 2005). Hsp70 could protect cells from stress-induced caspase-dependent apoptosis (Parcellier et al., 2003). To determine whether Hsp70 protected against PCV2-induced apoptosis, cell lysates were harvested at various time points and assayed for caspase-3 activity. Following infection with PCV2 alone or inoculated with rAd-wt, a time-dependent increase in the cleavage of pNA (a product of caspase-3 cleaving Ac-DEVD-pNA) was seen throughout

the post-infection time course. This indicated that caspase-3 was progressively activated by PCV2 infection (Fig. 9). Following inoculation with rAd-Hsp70 of PCV2-infected cells, caspase-3 activity increased up to 48 h post inoculation and then abruptly declined (Fig. 9). This showed the PCV2 caspase activation was blocked by the recombinant adenovirus expressing hsp70 at 72 h post inoculation. The protective effect was provided by Hsp70 against apoptosis.

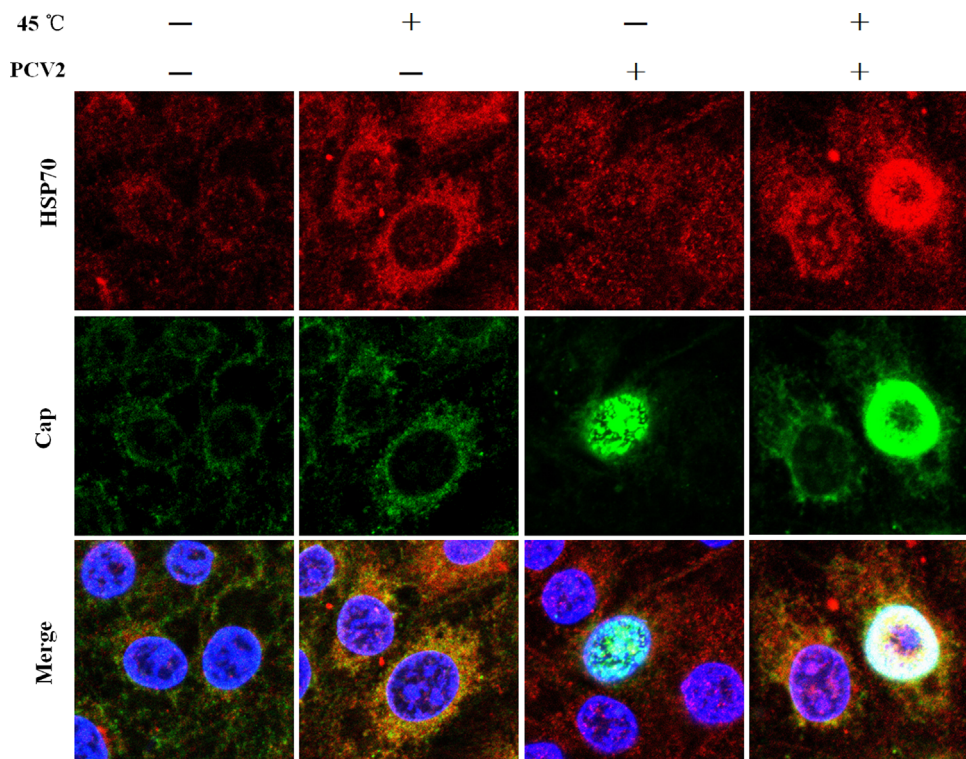


Fig. 6. Hsp70 colocalized with PCV2 Cap protein in 3D4/31 cells. Under heat shock and PCV2-only conditions, Cap protein and Hsp70 were detected with mouse and rabbit antibodies. Primary antibodies were detected with secondary antibodies conjugated to Alexa Fluor 488 or Alexa Fluor 555. Cells were examined by laser scanning confocal microscopy. PCV2-infected cells costained with Cap-specific mouse mAb (green) and Hsp70-specific rabbit polyclonal antibody (red). Yellow regions are areas of Cap protein and Hsp70 colocalization. Nuclei were stained using DAPI (blue).

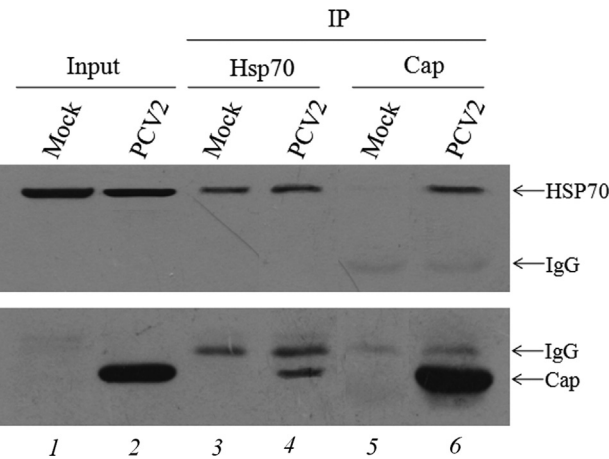


Fig. 7. Hsp70-Cap interaction by immunoprecipitation. 3D4/31 cells were subjected to heat shock after PCV2 infection. At 48 h after HS treatment, cells were harvested and lysed. The input samples were incubated with monoclonal antibodies against Hsp70 or Cap. Immune complexes were analyzed by Western blot using anti-Hsp70 or anti-Cap. Bands corresponding to Hsp70, Cap, and IgG are indicated.

Discussion

PCV2 is found worldwide in pigs and is linked to several pathological conditions collectively named porcine circovirus diseases (Allan and Ellis, 2000; Allan et al., 1998; Gillespie et al., 2009). Reactive oxygen species (ROS) promote PCV2 replication in PK-15 cells and LPS induces PCV2 replication in swine alveolar macrophages (Chang et al., 2006; Chen et al., 2012). Heat shock conditions enhance the infection of monocytic cells by dengue virus, both at penetration and post-penetration levels (Chavez-Salinas et al., 2008). Caloric stress

increases the susceptibility of BHK cells to rotavirus infection by more than 100-fold (Lopez et al., 2006). In this study, we firstly found that heat shock could effectively increase PCV2 production in the 3D4/31 cell line both in protein and DNA levels. The downregulation of Hsp70, using specific chaperons inhibitors such as quercetin or RNA interference, significantly reduced the intracellular level of viral proteins and viral progeny production. The confocal microscopy and co-immunoprecipitation results showed that Hsp70 could interact with the Cap protein of PCV2 during the virus replication. Meanwhile, the NF- κ B pathway was activated and caspase-3 activity was reduced during the virus replication. It indicated that heat shock was an effective inducer of PCV2 in cultured 3D4/31 cells. Hsp70 is involved in the viral transcription, translation, and/or production during the virus replication in the cell line after heat shock.

The heat shock response or stress response is the most highly conserved cellular response in all species. HSPs play important roles in antigen presentation and intracellular trafficking and apoptosis and act as molecular chaperones by helping nascent polypeptides assume their proper conformations (Khar et al., 2001). Activation of the heat-shock response is a specific virus function that ensures proper synthesis of viral proteins and virions, thus stress proteins may be important for virus replication (Glutzer et al., 2000). In order to understand the pathogenesis of PCV2, Ramirez-Boo et al. (2011) performed a proteomics study on inguinal lymph nodes of piglets inoculated with PCV2 using two proteomics strategies, 2-DE and 1-DE followed by 16O/18O peptide labeling and peptide identification and quantification by MS, and found that several heat shock proteins were altered by PCV2 infection, including hsp70 which was down-regulated. But our previously study showed that Hsp70 expression was up-regulated in PAMs at 12–48 h post infection of PCV2, which detected by using two-dimensional liquid chromatography–tandem mass spectrometry coupled with isobaric tags for relative and absolute quantification (iTRAQ) labeling approach (Liu et al., 2013).

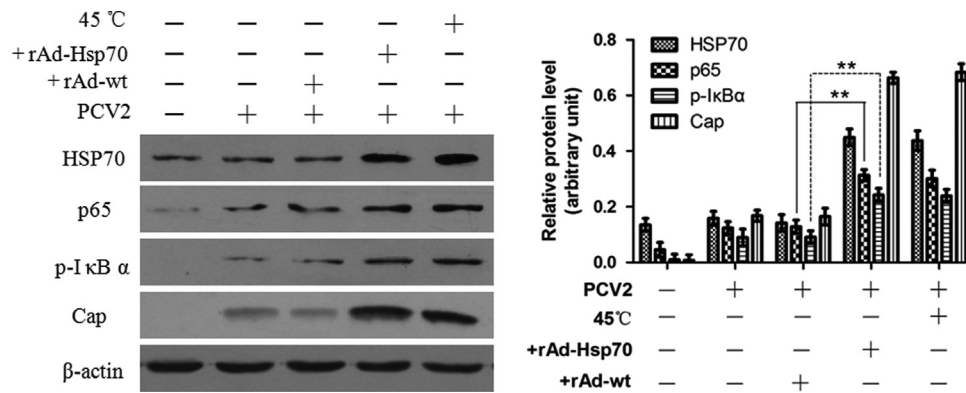


Fig. 8. Effect of rAd-hsp70 treatment on PCV2 replication and activation of NF- κ B. 3D4/31 cells were inoculated with rAd-hsp70 after PCV2 infection or treated with heat shock. Cells were lysed and extracts analyzed by Western blot using anti-Hsp70, anti-NF- κ B (p65), anti-p-I κ B α , anti-Cap, or anti- β actin.

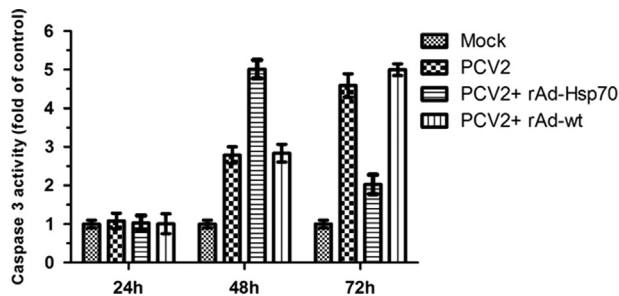


Fig. 9. Effect of Hsp70 overexpression on PCV2 replication and activation of caspase-3. Cell lysates were harvested at the indicated times post inoculation with rAd-hsp70. Caspase-3 activity was measured by a colorimetric assay based on the ability of caspase-3 to convert Ac-DEVD-pNA into a yellow formazan product. Shown are means from duplicated experiments.

In this study, our results showed that Hsp70 expression did not change greatly in 3D4/31 cell line at 37 °C post infection with PCV2 (Fig. 3). However, when the Hsp70 gene was knocked down in the cells by transfection with the small interfering RNA targeting to Hsp70 gene, the replication of PCV2 was significantly decreased after treatment with heat-shock (Fig. 4). Furthermore, the confocal microscopic analysis results showed that Hsp70 and Cap protein interacted and co-localized in the nuclear zone of the cells after heat-shock or Hsp70 overexpression. The interaction of Hsp70 and Cap protein might be beneficial for viral protein synthesis and replication. By the way, it should be noted that the 3D4/31 cell is an immortalized porcine macrophage cell line by SV40 large T antigen. PCV2 can replicate in the cells, but the replication capacity is very low. It should be important to further verify some of the key findings from this study in other cell culture systems such as PAM and PK-15 cells, which support much high level of PCV2 replication.

Adenoviruses can infect many host cells. The viral vector expression cassettes can deliver gene easily into cells with high efficiency and long duration. However, adenovirus itself could activate the IFN- β and decrease virus replication (Cavanaugh et al., 1998; Shiver et al., 2002). In order to determine whether Hsp70 was essential to sustain a high level of virus replication, we employed rAd-mediated Hsp70 gene expression. The results showed that rAd-mediated gene delivery specifically increased the intracellular Hsp70 levels in PCV2-infected cells and resulted in significant increases in virion production, compared to those in the wild type adenovirus control group. Meanwhile, the level of the recombinant adenovirus rAd-HSP70 replication was almost the same as that of wild type adenovirus by using a real-time PCR (data not shown here). It indicated that the recombinant

adenovirus expressing porcine HSP70 gene could be used to analogy the role of HSP in primary monocyte/macrophage lineage cells and in vivo toward PCV2 infection in the future.

NF- κ B is activated in response to cellular stress, including heat shock (Santoro, 2000). Chen et al. (2012) found that the generation of ROS during PCV2 infection is involved in replication. This is associated with an alteration in NF- κ B activity induced by ROS. In this report, we described a correlation between NF- κ B activation, phosphorylation of I κ B α , and PCV2 replication induced by Hsp70. Our further investigation showed that NF- κ B p65 protein increased and I κ B α protein was phosphorylated over time upon Hsp70 overexpression. This indicated that the NF- κ B pathway was activated and involved in the replication of PCV2 in 3D4/31 cells. PCV2 infection induced apoptosis by activating caspase-3 pathways in PK-15 cells (Liu et al., 2006, 2005). Up-regulation of caspase 8 and 3 in the spleens of infected mice might be the mechanism through which PCV2 induced apoptosis in mice (Kiupel et al., 2005). In contrast, several other studies contradicted the role of apoptosis in PCV2 pathogenesis (Resendes et al., 2004; Krakowka et al., 2004, 2005; Sinha et al., 2012). There was an inverse relationship between amounts of PCV2 antigen and apoptosis in the thymus and most peripheral lymphoid tissues of the pigs naturally affected by PCVAD. The pigs with severe lymphoid depletion had less apoptosis (Resendes et al., 2004; Sinha et al., 2012). In this study, our caspase-3 activity assays showed that PCV2 infection as well as expression of viral protein induced apoptosis. However, when Hsp70 was overexpressed in the infected cells, we observed that caspase-3 activity declined over time, which showed that Hsp70 accumulation led to a reduction of PCV2-induced apoptotic caspase-3 activity.

Of course, heat shock is a complicated and multidimensional response that directly and strongly induces HSP synthesis. For example, cellular chaperone Hsp27 showed modification and reorganization during Herpes Simplex Virus Type 1 Infection (Mathew et al., 2009). Hsp90 is involved in the assembly and nuclear transport of viral RNA polymerase subunits and also is a stimulatory host factor involved in influenza virus RNA Synthesis (Momose et al., 2002; Naito et al., 2007). It has been reported hsp27 was down-regulated in PCV2-infected PK-15 cells (Fan et al., 2012; Zhang et al., 2009). And the cellular protein Hsp40 could interact with the Cap protein of PCV2 which detected by using a yeast two-hybrid approach (Finsterbusch et al., 2009). Our previous report showed that Hsp 90- α and Hsp27 was down-regulated in PCV2-infected PAMs in the later stage of infection by using iTRAQ labeling approach (Liu et al., 2013), which was similar to other reports by Cheng et al. (2012) who examined PCV2-infected PAMs with 2-DE followed by MALDI-TOF/TOF. Here, we also found that Hsp27 and Hsp90 up-regulated in heat shock condition during replication of PCV2. But the roles of Hsp27 and Hsp90 in the virus replication should be studied in details

in the future. In addition, since Hsp70 chaperones can survey the folding status of proteins and regulate the fundamental cellular processes such as signal transduction, apoptosis and innate immunity, more cellular protein changes related to PCV2 replication during the period the heat shock also should be studied in the future. The affect of HSP on PCV2 replication also need to be elucidated in piglets fatherly.

In summary, this study firstly found that Hsp70 had a positive regulatory effect on PCV2 infection cycle. Hsp70 could interact with the viral Cap protein and play important role in the viral transcription, translation, and/or production. It should be helpful for understanding the mechanism of replication and pathogenesis of PCV2 and development of novel antiviral therapies in the future.

Materials and methods

Virus stock and cell infection

PCV2 strain WG09 (GenBank accession no. GQ845027) used in this study was isolated from a pig farm in Shanghai, China in 2009. The virus stock was a fourth-passage cell culture prepared in PK-15 cells with a titer of 10^6 TCID₅₀/ml. The continuous porcine monocytic cell line 3D4/31, developed from porcine alveolar Mφ (Weingartl et al., 2002), was grown in RPMI 1640 (GIBCO, Invitrogen Corporation, CA) supplemented with 10% FBS (Invitrogen), 1% nonessential amino acids ($100\times$, GIBCO-BRL), and antibiotics (20 mg/ml penicillin and streptomycin).

3D4/31 cells were cultured in medium containing PCV2 at an MOI of 1.0. About 24 h later, infected cell monolayers were near 80–90% confluent.

Heat shock treatment

When infected 3D4/31 cells monolayers were near 80–90% confluency, culture supernatant was removed and new medium with 2% FBS was added. Cells were heat shocked at 45 °C for 10 h, and then cells were reincubated at 37 °C.

Treatment of cells with quercetin

Infected 3D4/31 cells were kept in RPMI 1640 containing 100 μM quercetin (Sigma) for 24 h before heat shock treatment. Quercetin was reconstituted in dimethyl sulfoxide (DMSO). Untreated cells were maintained in medium containing the same concentration of DMSO. Culture supernatant was removed and new medium with 2% FBS added. Cells were subjected to heat shock at 45 °C for 10 h, followed by 37 °C for 72 h.

Assay of infected cells by indirect fluorescence assay (IFA)

Infected cells were washed with PBS, fixed with cold acetone/methanol (1/1 v/v) for 20 min at −20 °C, and air-dried. Fixed cells were incubated with pig anti-PCV2 polyclonal antiserum (VMRD, USA) at 37 °C for 1 h, washed three times with PBST (0.05% Tween-20 in PBS, pH 7.4), and incubated with staphylococcal protein A conjugated to FITC (Boshide, Wuhan, China) at 37 °C for 1 h in the dark. After three washes with PBST, infected cells were quantified by Zeiss LSM510 laser confocal microscopy. Fluorescence value was measured at 495 nm for excitation and 525 nm for emission with a fluorescence plate reader (TECAN infinite M200, TECAN).

Virus titration by IFA

The titre of the PCV2 sample was determined by IFA in 96-well plates. Briefly, 100 μl cell suspensions were inoculated into

semiconfluent monolayers of PK-15 cells and examined 72 h later for infection using IFA, according to standard methods (McNair et al., 2004).

Flow cytometry analysis

3D4/31 cells in 6-well plates were detached and washed once with PBS. Suspension staining of intracellular PCV2 antigens used commercially available fixation and permeabilization reagents (An Der Grub Fix&Perm Kit, Invitrogen, Austria). Cells were incubated with mAb to PCV2 Cap protein (made in our lab) at 37 °C for 1 h. After washing, cells were stained with Alexa Fluor 488-labeled goat anti-mouse IgG (Beyotime Biotech, Nantong, China) for 30 min. Unbound secondary antibody was washed away with PBS. Cells were resuspended in 200 μl PBS, and analyzed on a FACSCalibur cytometer (Becton Dickinson Immunocytometry Systems). At least 10,000 cells were analyzed for each sample.

siRNA experiments

All siRNAs (Invitrogen) were transfected with Lipofectamine 2000 transfection reagent (Invitrogen) according to the manufacturer's instructions. PCV2 infected 3D4/31 cells (in serum-free RPMI 1640) were transfected with 400 pmol/well of siRNA-Hsp70 or nonspecific siRNA. After 6 h at 37 °C, the medium was completed with 2%FCS. At 48 h post-transfection, cells were subjected to heat shock. Then cells were reincubated at 37 °C. Levels of the Hsp70 protein and viral production after siRNA transfection were analyzed by Western blotting and quantitative RT-PCR. The sequences were the following: siRNA-1Hsp70 (si1), 5'-UCGUCGAUGGUGCUG ACCAAGAUGA-3'; siRNA-2Hsp70 (si2), 5'-CAGACGGAGAUUCUACACGACGUACU-3'; siRNA-3Hsp70 (si3), 5'-AGGACUUCGACAACAGGCUGGUGAA-3'; nonspecific control (ns si), 5'-AUGGUGUCGUCGAAGAUGACUGACC-3' (scrambled S1 oligonucleotides).

Quantitative RT-PCR assays

Total DNA from cultured cells was isolated using the TaKaRa DNA Mini kit (TaKaRa, China) following the manufacturer's instructions, and assayed by real-time PCR as described (Feng et al., 2008). The sense primer PCV2F: 5'-CCAGAGGGCGTCTGACT-3', the antisense primer PCV2R: 5'-CGTTACCGTGGAGAAGGAA-3' and the probe 5'-FAM-AATGGCATCTTCAACACCCGCTC-TAMAM-3' were used to amplify PCV2 DNA. Quantitative real-time PCR was carried out using an ABI7300 v.1.3 (ABI).

Total RNA was extracted from the cellular samples using TRIzol reagent (Invitrogen). Reverse transcription was carried out using M-MLV Reverse Transcriptase (Promega) according to the Manufacturer's instructions as described previously (Li et al., 2009). Two microliters of the RT reaction mixture was submitted to quantitative RT-PCR (Q-PCR) using Hsp70 specific primers (sense: 5'-GCCGAGAAGGACGAGTTGA-3'; antisense: 5'-TGGTACAGTCCGCTGATGATG-3'), or β-actin (sense: 5'-TCTTCCAGCCCTCCTTCCT-3'; antisense: 5'-ACGTGCGACTTCATGATCGA-3'), and SYBR Green Real-time PCR Master Mix (Toyobo Co., Ltd., Osaka, Japan), according to the manufacturer's recommendations. The reaction procedure was 95 °C for 2 min, followed by 40 cycles at 95 °C for 15 s and 60 °C for 60 s. Cycle times of the internal reference that varied by > 1.0 unit in triplicate were discarded. The relative amount of target gene mRNA was normalized to that of β-actin mRNA in the same sample. To confirm specific amplification, melting curve analysis of the RT-PCR products was performed according to the manufacturer's protocol. The Q-PCR was performed in an ABI PRISM 7300 sequence detection system and analyzed with ABI PRISM 7300 SDS software (Applied Biosystems).

Western blot

3D4/31 cells grown in 24-well plates and lysed at the indicated times. Protein concentration was determined with the Pierce BCA Protein Assay Kit (Thermo Scientific, Product No. 23227, USA). Equivalent protein amounts of cell lysate were subjected to 12% SDS-PAGE gels and transferred to 0.22 μ m nitrocellulose membranes (Hybond-C extra, Amersham Biosciences). After blotting, membranes were incubated at 37 °C for 60 min with mouse monoclonal antibodies (mAbs) to actin (Abcam, Cambridge, U.K.), Hsp90 (Abcam, Cambridge, U.K.), phosphorylated (p)-I κ B α (Assaydesigns, Ann Arbor, MI, USA), PCV2 Cap protein (made in our lab), or rabbit polyclonal antibody to NF- κ B p65 (Santa Cruz Biotechnology, California, USA), Hsp70 (Santa Cruz Biotechnology, California, USA), Hsp27 (Santa Cruz Biotechnology, California, USA). After washing three times with 0.05% PBST, membranes were incubated at 37 °C for 60 min with horseradish peroxidase-conjugated goat anti-mouse IgG (Boshide, Wuhan, China) or goat anti-rabbit IgG (Boshide, Wuhan, China). Detection was performed using chemiluminescence luminol reagents (SuperSignalWest PicoTiter Kit, Pierce). Protein spot levels were determined by using ImageJ quantification software.

Recombinant adenovirus-mediated Hsp70 expression

Recombinant adenoviruses expressing intact hsp70 (GenBank accession No. EU693116) were constructed by our lab. Infected 3D4/31 cells were inoculated with rAd-hsp70 at MOI of 500 and rAd-wt as control. After 24 h, culture supernatant was removed and new medium with 2% FBS was added. After 72 h, IFA, real-time PCR and Western blotting were used to detect the effect of overexpressed hsp70 on PCV2 replication.

Confocal microscopy

Infected or mock-infected 3D4/31 cells were cultured on glass coverslips. Cells were processed for indirect immunofluorescence at 48 h post heat shock treatment by fixing in 4% paraformaldehyde for 30 min. Cell membranes were permeabilized by incubating with PBS buffer containing 0.2% Triton X-100 for 5 min. Samples were blocked in 1% BSA for 30 min before incubation with primary and secondary antibodies. All washes used PBS. PCV2 Cap protein was detected with mouse mAb anti-Cap protein at a 1:200 dilution. Hsp70 was detected with rabbit polyclonal antibody anti-hsp70 (Santa Cruz, California, USA) at a 1:500 dilution. Secondary antibodies were Alexa Fluor 488-conjugated goat anti-mouse (Beyotime) and Alexa Fluor 555-conjugated goat anti-rabbit (Beyotime). Coverslips were mounted onto microscope slides and samples were examined using a Zeiss LSM700 confocal microscope.

Immunoprecipitation assay

At 48 h post heat shock treatment, mock-infected cells or PCV2-infected cells were lysed in 1% nonidet P-40 (NP-40) lysis buffer (1% NP-40, 50 mM Tris-HCl (pH 7.4), 150 mM NaCl, and 2 mM EDTA) containing complete protease inhibitors (Sigma) on ice for 5 min. Lysates were cleared by centrifugation (10,000 \times g), and supernatants were incubated with Hsp70/Cap antibody overnight at 4 °C with rotation, 50% suspension protein G-Sepharose (Beyotime Biotech, Nantong, China) was added and cells were incubated for 3 h at 4 °C. Beads containing protein complexes were washed by three times with lysis buffer and complexes were eluted by boiling for 5 min in 2 \times gel sample buffer. Immunoprecipitated proteins were analyzed by Western immunoblotting.

Assay for NF- κ B pathway and caspase-3 activity

PCV2-infected 3D4/31 cells were collected at 24, 48 and 72 h post inoculation (hpi) with rAd-hsp70. Samples were used for NF- κ B pathway and caspase-3 activity assays.

Western blotting for the NF- κ B pathway component in PCV2 replication used rabbit polyclonal antibody to NF- κ B p65 (Santa Cruz, California, USA) and monoclonal antibody to phosphorylated (p)-I κ B α (Assaydesigns, Ann Arbor, MI, USA).

Caspase-3 activity was determined by a colorimetric assay based on the ability of caspase-3 to convert acetyl-Asp-Glu-Val-Asp *p*-nitroanilide (Ac-DEVD-pNA) into a yellow formazan product (*p*-nitroanilide). An increase in absorbance at 405 nm was used to quantify activation of caspase-3. Supernatants of medium and 3D4/31 cells were collected at indicated times. Cells were rinsed with cold PBS, and lysed with lysis buffer (100 μ L/2 \times 10⁶ cells) for 15 min on ice. Cell lysates were centrifuged at 18,000 \times g for 10 min at 4 °C. Caspase-3 activity was determined using caspase-3 activity kit (Beyotime Institute of Biotechnology, Nantong, China) following the manufacturer's protocol.

Statistical analysis

Statistical analysis was performed using GraphPad PRIM software (version 5.02 for Windows; GraphPad software Inc.). Data were analyzed to establish their significance using one-way or two-way analysis of variance (ANOVA) followed by a least-significant difference test. Data are expressed as means \pm SD. Differences were regarded as significant at $P < 0.01$ (**).

Acknowledgments

This work was supported by the special fund for agroscientific research in the public interest (201003060-4, 201203039), the Jiangsu Province Science and Technology Support Program (BE2012368), key grant of ministry of education of China (313031), the China agricultural research system foundation (CARS-36), and the Priority Academic Program Development of Jiangsu higher education institutions (PAPD).

References

- Allan, G.M., Ellis, J.A., 2000. Porcine circoviruses: a review. *J. Vet. Diagn. Invest.* 12, 3–14.
- Allan, G.M., McNeilly, F., Ellis, J., Krakowka, S., Botner, A., McCullough, K., Nauwynck, H., Kennedy, S., Meehan, B., Charreyre, C., 2004. PMWS: experimental model and co-infections. *Vet. Microbiol.* 98, 165–168.
- Allan, G.M., McNeilly, F., Kennedy, S., Daft, B., Clarke, E.G., Ellis, J.A., Haines, D.M., Meehan, B.M., Adair, B.M., 1998. Isolation of porcine circovirus-like viruses from pigs with a wasting disease in the USA and Europe. *J. Vet. Diagn. Invest.* 10, 3–10.
- Cavanaugh, V.J., Guidotti, L.G., Chisari, F.V., 1998. Inhibition of hepatitis B virus replication during adenovirus and cytomegalovirus infections in transgenic mice. *J. Virol.* 72, 2630–2637.
- Chae, C., 2005. A review of porcine circovirus 2-associated syndromes and diseases. *Vet. J.* 169, 326–336.
- Chang, H.W., Pang, V.F., Chen, L.J., Chia, M.Y., Tsai, Y.C., Jeng, C.R., 2006. Bacterial lipopolysaccharide induces porcine circovirus type 2 replication in swine alveolar macrophages. *Vet. Microbiol.* 115, 311–319.
- Chavez-Salinas, S., Ceballos-Olvera, I., Valle, J.R.D., Medina, F., del Angel, R.M., 2008. Heat shock effect upon dengue virus replication into U937 cells. *Virus Res.* 138, 111–118.
- Chen, X., Ren, F., Hesketh, J., Shi, X., Li, J., Gan, F., Huang, K., 2012. Reactive oxygen species regulate the replication of porcine circovirus type 2 via NF-kappaB pathway. *Virology* 426, 66–72.
- Cheng, S., Zhang, M., Li, W.T., Wang, Y., Liu, Y.Y., He, Q.G., 2012. Proteomic analysis of porcine alveolar macrophages infected with porcine circovirus type 2. *J. Proteomics* 75, 3258–3269.
- Cino-Ozuna, A.G., Henry, S., Hesse, R., Nietfeld, J.C., Bai, J., Scott, H.M., Rowland, R.R., 2011. Characterization of a new disease syndrome associated with porcine circovirus type 2 in previously vaccinated herds. *J. Clin. Microbiol.* 49, 2012–2016.

- Demarco, A., Santoro, M.G., 1993. Antiviral effect of short hyperthermic treatment at specific stages of vesicular stomatitis-virus replication cycle. *J. Gen. Virol.* 74, 1685–1690.
- Diaz-Latoud, C., Diaz, J.J., Fabre-Jonca, N., Kindbeiter, K., Madjar, J.J., Arrigo, A.P., 1997. Herpes simplex virus Us11 protein enhances recovery of protein synthesis and survival in heat shock treated HeLa cells. *Cell Stress Chaperon.* 2, 119–131.
- Fan, H.Y., Ye, Y., Luo, Y.W., Tong, T.Z., Yan, G.R., Liao, M., 2012. Quantitative proteomics using stable isotope labeling with amino acids in cell culture reveals protein and pathway regulation in porcine circovirus type 2 infected PK-15 cells. *J. Proteome Res.* 11, 995–1008.
- Feng, Z., Jiang, P., Wang, X., Li, Y., Jiang, W., 2008. Adenovirus-mediated shRNA interference against porcine circovirus type 2 replication both in vitro and in vivo. *Antiviral Res.* 77, 186–194.
- Finsterbusch, T., Steinfeldt, T., Doberstein, K., Rodner, C., Mankertz, A., 2009. Interaction of the replication proteins and the capsid protein of porcine circovirus type 1 and 2 with host proteins. *Virology* 386, 122–131.
- Gillespie, J., Opriessnig, T., Meng, X.J., Pelzer, K., Buechner-Maxwell, V., 2009. Porcine circovirus type 2 and porcine circovirus-associated disease. *J. Vet. Intern. Med.* 23, 1151–1163.
- Glötzel, J.B., Saltik, M., Chiocci, S., Michou, A.I., Moseley, P., Cotten, M., 2000. Activation of heat-shock response by an adenovirus is essential for virus replication. *Nature* 407, 207–211.
- Grau-Roma, L., Fraile, L., Segales, J., 2011. Recent advances in the epidemiology, diagnosis and control of diseases caused by porcine circovirus type 2. *Vet. J.* 187, 23–32.
- He, J., Cao, J., Zhou, N., Jin, Y., Wu, J., Zhou, J., 2012. Identification and functional analysis of the novel ORF4 protein encoded by porcine circovirus type 2. *J. Virol.*
- Hirayama, E., Atagi, H., Hiraki, A., Kim, J., 2004. Heat shock protein 70 is related to thermal inhibition of nuclear export of the influenza virus ribonucleoprotein complex. *J. Virol.* 78, 1263–1270.
- Jindal, S., Young, R.A., 1992. Vaccinia virus infection induces a stress response that leads to association of Hsp70 with viral proteins. *J. Virol.* 66, 5357–5362.
- Khar, A., Ali, A.M., Pardhasaradhi, B.V., Varalakshmi, C.H., Anjum, R., Kumari, A.L., 2001. Induction of stress response renders human tumor cell lines resistant to curcumin-mediated apoptosis: role of reactive oxygen intermediates. *Cell Stress & Chaperon.* 6, 368–376.
- Kim, J., Ha, Y., Chae, C., 2006. Potentiation of porcine circovirus 2-induced postweaning multisystemic wasting syndrome by porcine parvovirus is associated with excessive production of tumor necrosis factor- α . *Vet. Pathol.* 43, 718–725.
- Kiupel, M., Stevenson, G.W., Galbreath, E.J., North, A., HogenEsch, H., Mittal, S.K., 2005. Porcine circovirus type 2 (PCV2) causes apoptosis in experimentally inoculated BALB/c mice. *BMC Vet. Res.* 1, 7.
- Krakowka, S., Ellis, J., McNeilly, F., Meehan, B., Oglesbee, M., Alldinger, S., Allan, G., 2004. Features of cell degeneration and death in hepatic failure and systemic lymphoid depletion characteristic of porcine circovirus-2-associated postweaning multisystemic wasting disease. *Vet. Pathol.* 41, 471–481.
- Krakowka, S., Ellis, J., McNeilly, F., Waldner, C., Allan, G., 2005. Features of porcine circovirus-2 disease: correlations between lesions, amount and distribution of virus, and clinical outcome. *J. Vet. Diagn. Invest.* 17, 213–222.
- Li, G., Jiang, P., Li, Y., Wang, X., Huang, J., Bai, J., Cao, J., Wu, B., Chen, N., Zeshan, B., 2009. Inhibition of porcine reproductive and respiratory syndrome virus replication by adenovirus-mediated RNA interference both in porcine alveolar macrophages and swine. *Antiviral Res.* 82, 157–165.
- Liu, J., Bai, J., Lu, Q., Zhang, L., Jiang, Z., Michal, J.J., He, Q., Jiang, P., 2013. Two-dimensional liquid chromatography–tandem mass spectrometry coupled with isobaric tags for relative and absolute quantification (iTRAQ) labeling approach revealed first proteome profiles of pulmonary alveolar macrophages infected with porcine circovirus type 2. *J. Proteomics* 79, 72–86.
- Liu, J., Chen, I., Du, Q., Chua, H., Kwang, J., 2006. The ORF3 protein of porcine circovirus type 2 is involved in viral pathogenesis in vivo. *J. Virol.* 80, 5065–5073.
- Liu, J., Chen, I., Kwang, J., 2005. Characterization of a previously unidentified viral protein in porcine circovirus type 2-infected cells and its role in virus-induced apoptosis. *J. Virol.* 79, 8262–8274.
- Lopez, T., Lopez, S., Arias, C.F., 2006. Heat shock enhances the susceptibility of BHK cells to rotavirus infection through the facilitation of entry and post-entry virus replication steps. *Virus Res.* 121, 74–83.
- Mankertz, A., Mankertz, J., Wolf, K., Buhk, H.J., 1998. Identification of a protein essential for replication of porcine circovirus. *J. Gen. Virol.* 79 (Pt 2), 381–384.
- Mathew, S.S., Della Selva, M.P., Burch, A.D., 2009. Modification and reorganization of the cytoprotective cellular chaperone Hsp27 during herpes simplex virus type 1 infection. *J. Virol.* 83, 9304–9312.
- Mayer, M.P., 2010. Gymnastics of molecular chaperones. *Mol. Cell.* 39, 321–331.
- Mayer, M.P., Bukau, B., 2005. Hsp70 chaperones: cellular functions and molecular mechanism. *Cell. Mol. Life Sci.* CMLS 62, 670–684.
- McNair, I., Marshall, M., McNeilly, F., Botner, A., Ladekjaer-Mikkelsen, A.S., Vincent, I., Herrmann, B., Sanchez, R., Rhodes, C., 2004. Interlaboratory testing of porcine sera for antibodies to porcine circovirus type 2. *J. Vet. Diagn. Invest.* 16, 164–166.
- Momose, F., Naito, T., Yano, K., Sugimoto, S., Morikawa, Y., Nagata, K., 2002. Identification of Hsp90 as a stimulatory host factor involved in influenza virus RNA synthesis. *J. Biol. Chem.* 277, 45306–45314.
- Morimoto, R.I., 1991. Heat shock: the role of transient inducible responses in cell damage, transformation, and differentiation. *Cancer Cells* 3, 295–301.
- Nagy, P.D., Wang, R.Y., Pogany, J., Hafren, A., Makinen, K., 2011. Emerging picture of host chaperone and cyclophilin roles in RNA virus replication. *Virology* 411, 374–382.
- Naito, T., Momose, F., Kawaguchi, A., Nagata, K., 2007. Involvement of Hsp90 in assembly and nuclear import of influenza virus RNA polymerase subunits. *J. Virol.* 81, 1339–1349.
- Nawagatgul, P., Morozov, I., Bolin, S.R., Harms, P.A., Sorden, S.D., Paul, P.S., 2000. Open reading frame 2 of porcine circovirus type 2 encodes a major capsid protein. *J. Gen. Virol.* 81, 2281–2287.
- Parcellier, A., Gurbuxani, S., Schmitt, E., Solary, E., Garrido, C., 2003. Heat shock proteins, cellular chaperones that modulate mitochondrial cell death pathways. *Biochem. Biophys. Res. Commun.* 304, 505–512.
- Patterson, A.R., Opriessnig, T., 2010. Epidemiology and horizontal transmission of porcine circovirus type 2 (PCV2). In: *Animal Health Research Reviews/Conference of Research Workers in Animal Diseases* 11, pp. 217–234.
- Ramirez-Boo, M., Nunez, E., Jorge, I., Navarro, P., Fernandes, L.T., Segales, J., Garrido, J.J., Vazquez, J., Moreno, A., 2011. Quantitative proteomics by 2-DE, 160/180 labelling and linear ion trap mass spectrometry analysis of lymph nodes from piglets inoculated by porcine circovirus type 2. *Proteomics* 11, 3452–3469.
- Resendes, A.R., Majo, N., Segales, J., Mateu, E., Calsamiglia, M., Domingo, M., 2004. Apoptosis in lymphoid organs of pigs naturally infected by porcine circovirus type 2. *J. Gen. Virol.* 85, 2837–2844.
- Santoro, M.G., 1994. Heat shock proteins and virus replication: hsp70s as mediators of the antiviral effects of prostaglandins. *Experientia* 50, 1039–1047.
- Santoro, M.G., 2000. Heat shock factors and the control of the stress response. *Biochem. Pharmacol.* 59, 55–63.
- Segales, J., Allan, G.M., Domingo, M., 2005. Porcine circovirus diseases. In: *Animal Health Research Reviews/Conference of Research Workers in Animal Diseases* 6, pp. 119–142.
- Segales, J., Sitjar, M., Domingo, M., Dee, S., Del Pozo, M., Noval, R., Sacristan, C., De las Heras, A., Ferro, A., Latimer, K.S., 1997. First report of post-weaning multisystemic wasting syndrome in pigs in Spain. *Vet. Rec.* 141, 600–601.
- Shiver, J.W., Fu, T.M., Chen, L., Casimiro, D.R., Davies, M.E., Evans, R.K., Zhang, Z.Q., Simon, A.J., Trigona, W.L., Dubey, S.A., Huang, L., Harris, V.A., Long, R.S., Liang, X., Handt, L., Schleif, W.A., Zhu, L., Freed, D.C., Persaud, N.V., Guan, L., Punt, K.S., Tang, A., Chen, M., Wilson, K.A., Collins, K.B., Heidecker, G.J., Fernandez, V.R., Perry, H.C., Joyce, J.G., Grimm, K.M., Cook, J.C., Keller, P.M., Kresock, D.S., Mach, H., Troutman, R.D., Isopi, L.A., Williams, D.M., Xu, Z., Bohannon, K.E., Volkin, D.B., Montefiori, D.C., Miura, A., Krivulka, G.R., Lifton, M.A., Kuroda, M.J., Schmitz, J.E., Letvin, N.L., Caulfield, M.J., Bett, A.J., Youil, R., Kaslow, D.C., Emini, E.A., 2002. Replication-incompetent adenoviral vaccine vector elicits effective anti-immunodeficiency-virus immunity. *Nature* 415, 331–335.
- Sinha, A., Schalk, S., Lager, K.M., Wang, C., Opriessnig, T., 2012. Singular PCV2a or PCV2b infection results in apoptosis of hepatocytes in clinically affected gnotobiotic pigs. *Res. Vet. Sci.* 92, 151–156.
- Sullivan, C.S., Pitas, J.M., 2001. The virus-chaperone connection. *Virology* 287, 1–8.
- Wei, L., Kwang, J., Wang, J., Shi, L., Yang, B., Li, Y., Liu, J., 2008. Porcine circovirus type 2 induces the activation of nuclear factor kappa B by IkappaBalpha degradation. *Virology* 378, 177–184.
- Weingartl, H.M., Sabara, M., Pasick, J., van Moorlehem, E., Babiuk, L., 2002. Continuous porcine cell lines developed from alveolar macrophages: partial characterization and virus susceptibility. *J. Virol. Methods* 104, 203–216.
- Zhang, X., Zhou, J.Y., Wu, Y.P., Zheng, X.J., Ma, G.P., Wang, Z.T., Jin, Y.L., He, J.L., Yan, Y., 2009. Differential proteome analysis of host cells infected with porcine circovirus type 2. *J. Proteome Res.* 8, 5111–5119.